Differentiation of iPSCs with the NGN2 construct into cortical neurons protocol

Christian Tarr[1], Claire Clelland[1, 2]

[1]University of California, San Francisco, Weill Institute for Neurosciences, San Francisco, CA, United States
[2]Contact: claire.clelland@ucsf.edu

October 23, 2025

This protocol is adapted from Wang and Ward *et. al.*, 2017.

This protocol describes the differentiation of induced pluripotent stem cells (iPSCs) into cortical neurons using a doxycycline-inducible Neurogenin 2 (NGN2) construct.

Table 1. Recommended reagent volume and seeding densities for standard cell culture vessels

Well Format	Matrigel Coating Volume	DPBS/ PDL	Accutase Volume	Media Volume	Suggested Day 0 Seeding Density/well	Suggested Day 3 Seeding Density/well
96-well	50uL	100uL	30ul	100uL	N/A	ICC: 10-20k Drug: 20-40k
24-well	250uL	250uL	125ul	500uL	N/A	100-250k
12-well	500uL	500uL	250ul	1mL	N/A	500-750k
6-well	1mL	1mL	500uL	2mL	750k	750k-1 million
10-cm dish	4mL	6mL	3mL	6mL	4 million	7 million
15-cm dish	12mL	18mL	9mL	18mL	N/A	20 million

Maintenance of iPSCs

1. Culture iPSCs changing the media every day, once cells are over 20% confluent.
2. Passage cells once they reach 60-75% confluency. Do not exceed 90% confluency
    a. Note: Cells should not be differentiated from thaw. Passage 1-3 times, or until cells reach growth phase and appear healthy (Fig.1A)
3. Before starting the differentiation, ensure you have the proper number of wells to differentiate, while keeping an additional well to maintain the cell line
    a. Note: Ensure wells have enough cells to differentiate, around 75% confluency

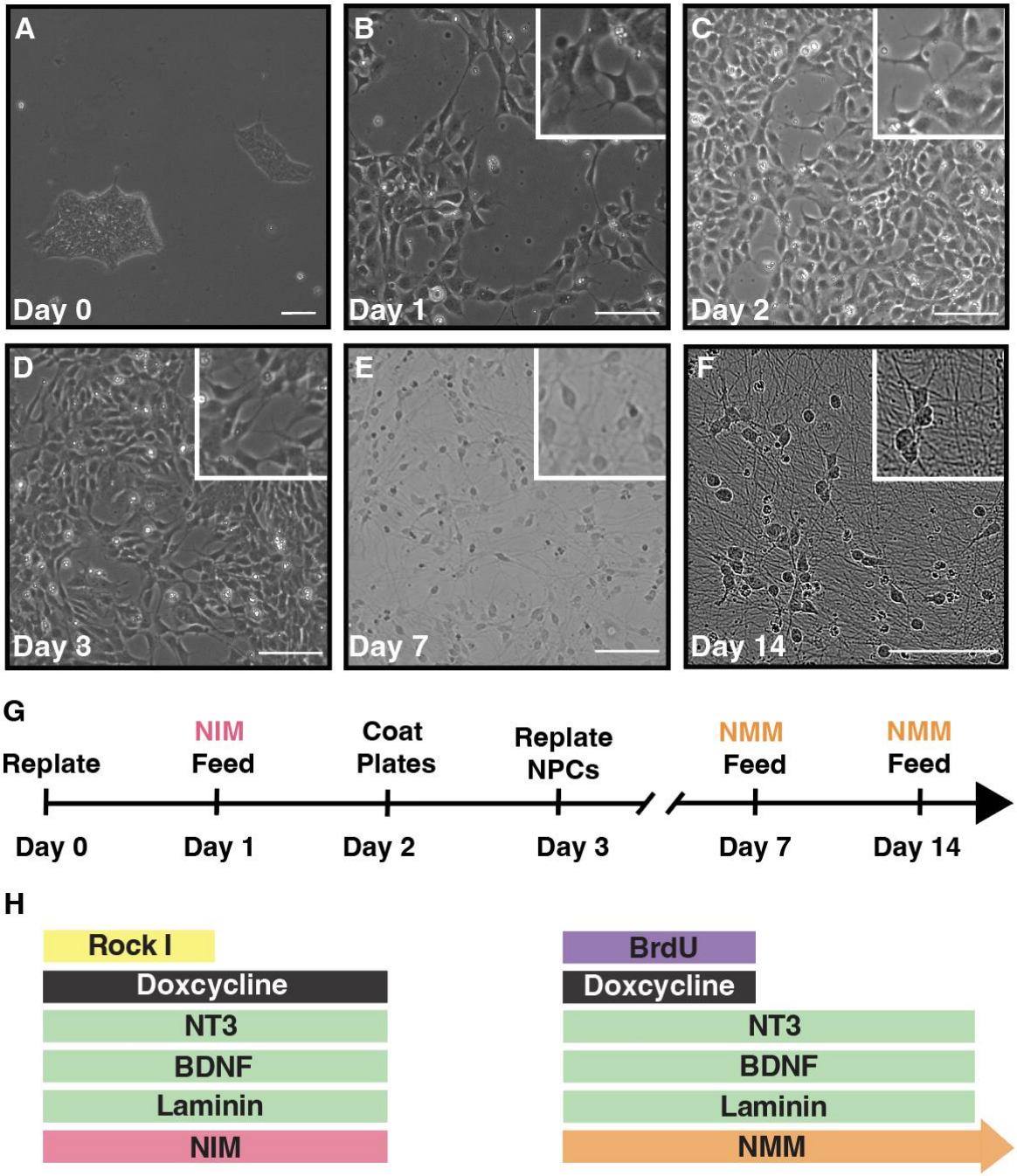

Figure 1. Timeline and overview of NGN2 differentiation. (A) iPSCs should appear healthy in large multicell colonies. (B) Early NPCs exhibit signs of neuritic extensions with “spikey” morphology and do not form homogeneous colonies. (C) Confluence nearly doubles with a similar morphology to day 1. (D) Plate is nearly 100% confluent with single cell NPCs that have defined, small neuritic processes. (E) Neuron morphology is apparent with clear somas and extended axon processes. (F) Neurons maintain defined somas with a robust axon network. (G) Timeline of NGN2 differentiation. (H) Neuronal media corresponding to each day within NGN2 timeline. Scale bars, 50um

NGN2 Day 0 Differentiation - *Replating iPSCs*

Media used

Table 2. Neural Induction Media (NIM)

Reagent	Media Dilution Factor	Stock Concentration	100mL Recipe
KO DMEM/F12	1	1X	100mL
N2 Supp.	1:100	100X	1mL
NEAA	1:100	100X	1mL

Table 3. Day 0 NGN2 Media

Reagent	Media Dilution Factor	Stock Concentration	50mL Recipe
NIM	1	1	50mL
Doxycycline*	1:1000	2mg/mL	50uL
Rock Inhibitor	1:1000	10mM	50uL
Laminin	1:1000	1mg/mL	50uL
BDNF	1:2000	20ug/mL	25uL
NT3	1:2000	20ug/mL	25uL

*Doxycycline is light sensitive and should be added immediately before use

NGN2 Day 0 Protocol

1. Matrigel coat plates (6-well or 10cm recommended) and incubate at 37°C for at least 30 mins or O/N
2. Prepare NIM then **Day 0 NGN2** media
    a. Note: Make enough NIM media for day 0 replating, day 1 feeding and cell resuspension. Store at 4°C for next day media change

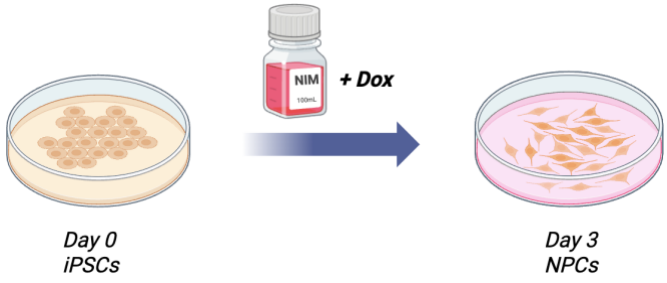

Figure 2. After 3 days in NIM + Dox iPSCs differentiate to neural progenitor cells (NPCs).

3. Aspirate media from iPSCs and wash with 1X DPBS
4. Add accutase and incubate at 37°C for 5-7 mins
5. Dissociate iPSCs by quenching with 1X DPBS at a volume 5 times that of accutase. Collect cell suspension in a 15mL or 50mL conical tube.
    a. Note: Less DPBS can be used to avoid overfilling the conical tube if pooling from multiple wells
6. Spin down cells at 300g for 3 minutes
7. Aspirate the supernatant and resuspend cells in **Day 0 NGN2** media
8. Count cells and calculate your desired cell amount (see Table 1 for suggestions)

$$\frac{\text{Number of cells needed (cells)}}{\text{Cell count (cells/mL)}} = \text{Cells to plate (mL)}$$

    a.
    b. Note: Cell count roughly doubles from day 0 to 3. Plate enough cells to account for all downstream experiments when replating on day 3
9. Add media to the well plate(s) being used and plate the calculated cell volume

NGN2 Day 1 Differentiation - *Feeding*

Media used

Table 4. Day 1 NGN2 media

Reagent	Media Dilution Factor	Stock Concentration	50mL Recipe
NIM	1	1	50mL
Doxycycline	1:1000	2mg/mL	50uL
Laminin	1:1000	1mg/mL	50uL
BDNF	1:2000	20ug/mL	25uL
NT3	1:2000	20ug/mL	25uL

NGN2 Day 1 Protocol

1. Prepare **Day 1 NGN2** media
    a. Note: Media should be prepared on the day of use only
2. Perform a full media change, being careful not to let the cell surface dry out

Figure 3. Day 1 full media change without rock inhibitor (RI)

NGN2 Day 2 Differentiation - *Coat Plates*

NGN2 Day 2 Protocol

1. No media change is required on day 2
2. Use 1X PDL to coat as many plates as needed and incubate at 37°C for at least 2 hours but ideally O/N

NGN2 Day 3 Differentiation - *Replate NPCs*

Media used

Table 5. Neural Maturation Media (NMM)

Reagent	Media Dilution Factor	Stock Concentration	100mL Recipe
DMEM/F-12	1:2	1X	50mL
Neurobasal-A	1:2	1X	50mL
NEAA	1:100	100X	1mL
GlutaMAX	1:100	100X	1mL
N2 Supplement	1:100	100X	1mL
B27 Supplement	1:50	50X	2mL

Table 6. Day 3 NGN2 media*

Reagent	Media Dilution Factor	Stock Concentration	50mL Recipe
NMM	1	1	50mL
Doxycycline	1:1000	2mg/mL	50uL
BrdU**	1:1000	40mM	50uL
Laminin	1:1000	1mg/mL	50uL
BDNF	1:2000	20ug/mL	25uL
NT3	1:2000	20ug/mL	25uL

*Rock inhibitor at 1:1000 can be added if the cell line or assay is prone to cell death
** BrdU is light sensitive and should be added immediately before use

NGN2 Day 3 Protocol

Part 1

1. Aspirate PDL from coated plates and wash 3 times with sterile distilled water
    a. Note: PDL is toxic and residual coating can be harmful to cultures
2. Let plates dry in the hood with the lid askew until the well surface is completely dry (30-60 mins).

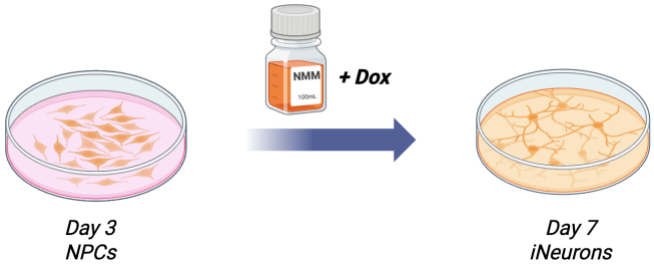

Figure 4. After 4 days in NMM + Dox NPCs fully differentiate into induced neurons (iNeurons).

Part 2

3. While plates dry, make **NMM** and **Day 3 NGN2** media
4. Once plates are dry, aspirate media from the neural progenitor cells (NPCs)
5. Wash cells with 1X DPBS
6. Add accutase and incubate at 37°C for 5-7 mins
    a. Note: NPCs tend to detach less readily from plates. Longer incubation times up to around 10 mins may be needed
7. Dissociate NPCs by quenching with 1X DPBS at a volume 5 times that of accutase. Collect cell suspension in a 15mL or 50mL conical tube.
8. Centrifuge the cells at **200** x g for 5 min.
9. Aspirate the supernatant and resuspend in **Day 3 NGN2** media
10. Count cells and calculate your desired cell amount:

$$\frac{\text{Number of cells needed (cells)}}{\text{Cell count (cells/mL)}} = \text{Cells to plate (mL)}$$

    a.
    b. Note: The number of NPCs plated depends on your specific experiment and desired end result. Experiments that you expect cell death due to treatment or require high extraction yields should use the higher cell density suggested in Table 1. Experiments where single cell resolution is necessary (e.g. ICC), use lower cell suggestion.
11. Add media to the well plate(s) being used and plate the calculated cell volume

NGN2 Day 7+ Differentiation

Media used

Table 7. Day 7 NGN2 media

Reagent	Media Dilution Factor	Stock Concentration	50mL Recipe
NMM	1	1	50mL
Laminin	1:1000	1mg/mL	50uL
BDNF	1:2000	20ug/mL	25uL
NT3	1:2000	20ug/mL	25uL

NGN2 Day 7+ Protocol

1. Prepare **Day 7 NGN2** media
2. Perform a full media change, being extra careful not to lift or dry out the cell surface
3. For the remainder of your experiment, use a weekly half-media change (e.g. day 14, day 21, etc.)

Troubleshooting

iPSC Spontaneous Differentiation

If iPSCs are unhealthy or spontaneously differentiating, NGN2 differentiation won't yield high-quality or quantity of induced neurons because alternate iPSC fates have already been chosen. Spontaneously differentiating cells take on a "spikey" or "cobblestone" appearance (Fig. 5B, C). If earlier passage numbers of the cell line are frozen down, it's usually faster to thaw new vials of the line than to clean a culture dominated by unhealthy cells. However, if the line is precious, these methods can be used to restore the iPSC line.

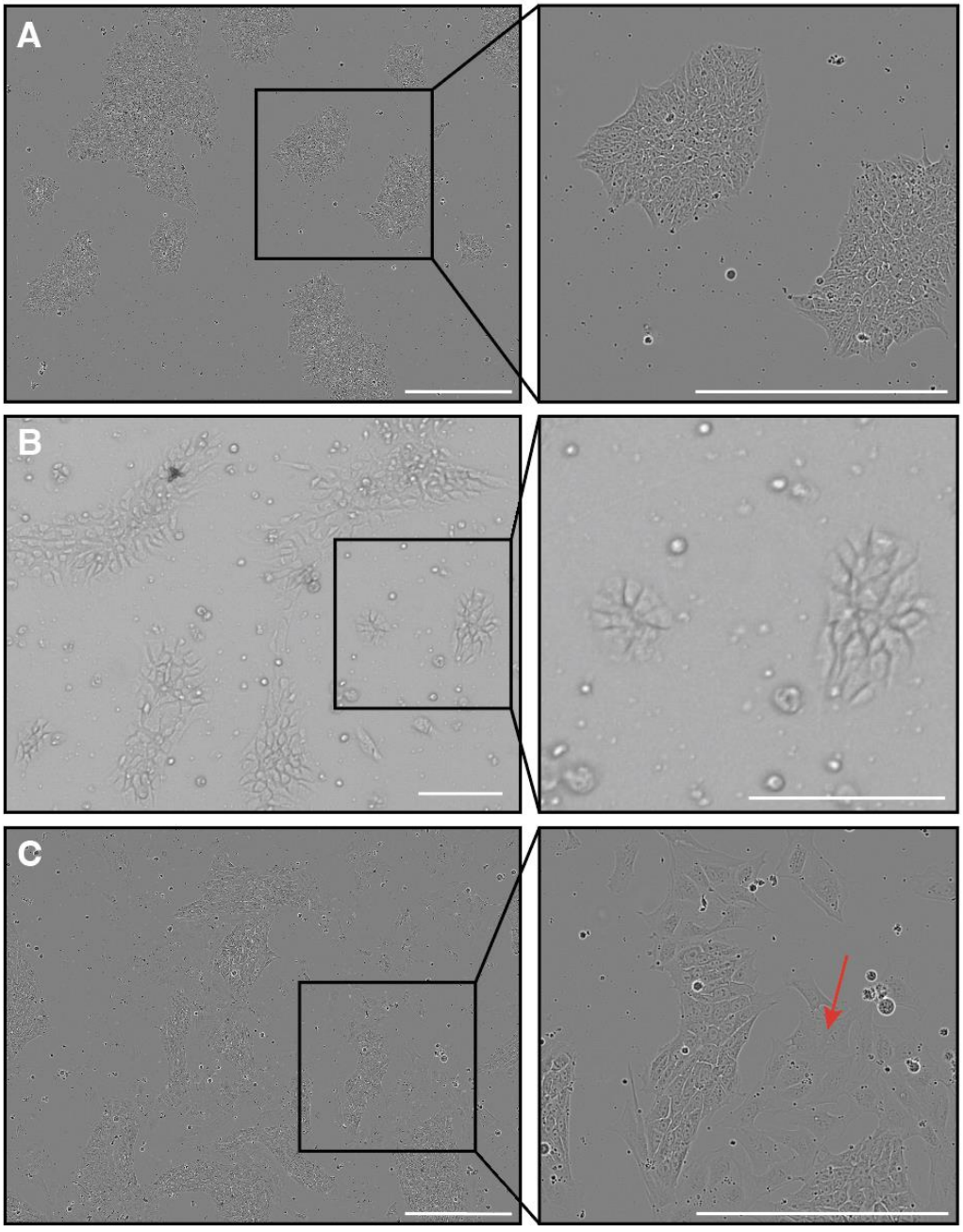

Figure 5. **Comparison of healthy and spontaneously differentiating cells.** (A) Healthy iPSCs with isolated multi-cell colonies. (B) Unhealthy iPSCs take on a “cobblestone” appearance. (C) Spontaneously differentiating iPSCs migrate away from colonies and appear flat with translucent cytoplasm and “spikey” cell membrane. Red arrow denotes spontaneously differentiated cells. Scale bars, 100 µm.

Light Passaging

1. iPSCs should be around 50% confluent, less than the normal confluence for standard line maintenance passaging
2. Matrigel coat 3 wells of a 6-well plate and incubate at 37 °C for 30 mins
3. Aspirate media from the iPSCs
4. Wash once with 1X DPBS
5. Add EDTA and incubate the plate at 37 °C for 2-3 minutes
    a. Note: Non-spontaneously differentiated or healthy cells tend to detach from the plate more readily, leaving behind unhealthy cells. Using a shorter incubation time can help select for healthier cells
6. Aspirate matrigel from 6-well plate and replace with media
7. Carefully aspirate the EDTA
8. Tilt the plate towards you and slowly add media to the well wall closest to you
9. Lay the plate flat and slowly tilt it back towards yourself to pool the media with your detached cells in the corner of the well
10. Collect the media that contains cells in suspension and plate in a gradient across the 3 wells of the 6-well plate
11. Return the plate to 37 °C

Colony Picking

1. If 1-3 rounds of light passaging do not eliminate spontaneous differentiation, a colony picking method can be used
2. iPSCs should be around 50% confluent or less in order to identify individual colonies
3. Use a phase contrast microscope, find healthy, isolated colonies in the well (see Fig. 5A)
4. Using a marker, lift up the well plate and dot the bottom of the well where the colony is
5. Use the microscope to ensure dot placement properly marks the colony
6. Find around 3-5 colonies, if possible
7. Matrigel coat a 48-well, 24-well, or 12-well, depending on the size and number of healthy colonies identified
8. Incubate matrigel coated plates for at least 30 mins at 37 °C
9. Aspirate media from the iPSCs
10. Wash once with 1X DPBS
11. Add EDTA and incubate the plate at 37 °C for 4-5 minutes
12. Aspirate the matrigel from coated plates and add media to the receiving plate
13. Carefully aspirate EDTA from the iPSC plate

14. Using a P2 pipette, load 1-2ul of media into the tip and carefully dispense and collect the media on the marked isolated colony
15. Deposit the media containing the colony into a single well of your plate
16. Repeat steps 14 and 15 until all marked colonies have been picked
17. Return the plate to 37 °C
18. As the cells grow, expand the line from the culture vessel used back into a 6-well format

Materials

Table 8. Reagents

Item	Manufacturer	Catalog Number
Knockout DMEM/F-12	ThermoFisher Scientific	12660012
Growth Factor Reduced Matrigel	Corning	356231
mTeSR™ Plus and supplement	Stemcell Technologies	1000276
Gibco™ DPBS, no Ca, no Mg	ThermoFisher Scientific	14-190-235
Accutase	Stemcell Technologies	07920
DMEM/F-12, HEPES	ThermoFisher Scientific	11330032
N-2 Supplement	ThermoFisher Scientific	17502048
MEM Non-Essential Amino Acids Solution (NEAA)	ThermoFisher Scientific	11140050
GlutaMAX Supplement	ThermoFisher Scientific	35050061
B-27 Supplement, serum free	ThermoFisher Scientific	17504044
Poly-D-Lysine (PDL)	ThermoFisher Scientific	A3890401
Laminin Mouse Protein, Natural	ThermoFisher Scientific	23017015
Doxycycline Hyclate (reconstituted in water)	Millipore Sigma	D3447
ROCK1 Inhibitor (Y-27632 2HCl)	Selleckchem	S1049

5-Bromo-2'-deoxyuridine (BrdU)	Millipore Sigma	B9285
Neurobasal-A	ThermoFisher Scientific	10888022
Recombinant Human BDNF	Peprotech	450-10
Recombinant Human NT3	Peprotech	450-03

Table 9. Plates

Well Format	Coating	Item	Manufacturer	Catalog Number
96-well	Standard	Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate with Lid	Corning	353219
	Pre-coated	Corning™ BioCoat™ 96-Well, Poly-D Lysine-Treated, Flat-Bottom Microplate	Corning	354640
24-well	Standard	Clear, Cellstar®, TC, Lid with Condensation Rings, Sterile, Single Packed	Greiner Bio-One	662160
	Pre-coated	Corning® BioCoat® Poly-D-Lysine 24-well Clear Flat Bottom TC-treated Multiwell Plate, with Lid	Corning	354414
12-well	Standard	Clear, Cellstar®, TC, Lid with Condensation Rings, Sterile, Single Packed	Greiner Bio-One	665180
	Pre-coated	Corning® BioCoat® Poly-D-Lysine 12-well Clear Flat Bottom TC-treated Multiwell Plate, with Lid	Corning	354470
6-well	Standard	Clear, Cellstar®, TC, Lid with Condensation Rings, Sterile, Single Packed	Greiner Bio-One	657160
	Pre-coated	Corning® BioCoat® Poly-D-Lysine 6-well Clear Flat Bottom TC-treated Multiwell Plate, with Lid	Corning	354413

Table 10. Equipment

Item	Manufacturer	Catalog Number
Eppendorf® Centrifuge 5810/5810R	Millipore Sigma	EP022628168
Invitrogen Countess™ II automated cell counter	ThermoFisher Scientific	AMQAX1000
Countess™ Cell Counting Chamber Slides and Holder, disposable	ThermoFisher Scientific	C10312

References

[1] Wang C, Ward ME, Chen R, Liu K, Tracy TE, Chen X, Xie M, Sohn PD, Ludwig C, Meyer-Franke A, Karch CM, Ding S, Gan L. Scalable Production of iPSC-Derived Human Neurons to Identify Tau-Lowering Compounds by High-Content Screening. Stem Cell Reports. 2017.

[2] Fernandopulle MS, Prestil R, Grunseich C, Wang C, Gan L, Ward ME. Transcription Factor-Mediated Differentiation of Human iPSCs into Neurons. Curr Protoc Cell Biol. 2018 Jun;79(1):e51. doi: 10.1002/cpcb.51. Epub 2018 May 18. PMID: 29924488; PMCID: PMC6993937.

[3] Merissa Chen*, Nina Draeger*, Martin Kampmann*, Kun Leng*, Emmy Li*, Connor Ludwig*, Greg Mohl*, Avi Samelson*, Syd Sattler*, Ruilin Tian*. 2020. Kampmann Lab iNeuron pre-differentiation & differentiation protocol. Protocols.io.